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A COMPARISON OF MIXED FLASK CULTURE AND STANDARDIZED LABORATORY MODEL ECOSYSTEMS FOR TOXICITY TESTING

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A COMPARISON OF MIXED FLASK CULTURE AND STANDARDIZED LABORATORY MODEL ECOSYSTEMS FOR TOXICITY TESTING

ABSTRACT: Two microecosystem protocols, the Standardized Aquatic Microcosm (SAM) method developed by Dr. Frieda Taub and associates (1982) and the Mixed Flask Culture (MFC) method of Dr. John Leffler (1981), were compared on the basis of their response to copper sulfate. These protocols differed in microcosm structure, age, and the variables monitored.

Two complete experiments were conducted with each method so that repeatability as well as sensitivity and variability could be evaluated. Copper caused significant reductions in pH, oxygen gain, and oxygen loss at concentrations down to 500 micrograms per litre (the lowest test concentration) in the SAM tests and 320 micrograms per litre in the MFC tests. Lower copper concentrations (36 and 70 micrograms per litre) in the MFC tests caused significant increases over control levels for several system level variables.

Although responses were similar with both systems, the SAM procedure provided considerably more insight into the changes in population densities and nutrient cycling responsible for the observed ecosystem level changes. This additional information was not obtained without cost, however. The SAM protocol was much more labor intensive and required about six times more laboratory effort than did the MFC protocol.

The SAM systems, on the basis of their higher coefficients of

variation (CV) and higher Minimum Detectable Differences (MDD), appeared to be theoretically less able to demonstrate significant toxic effects. This higher variability was at least partially offset by scheduling toxicant addition during the more sensitive early stages of microcosm development, since the frequency with which statistically significant effects were observed at comparable treatment levels suggested no inherent difference in sensitivity between the two systems.

The SAM results were more consistant, as microcosms developed and responded to copper in a similar manner in both runs. In contrast, the microcosms in the MFC experiments showed major differences in both development and sensitivity between runs.

Both methods were found to have strengths and weaknesses. The SAM protocol was more repeatable and provided a much more complete picture of toxicant effects, but was extremely labor intensive. The MFC protocol lacked repeatability and provided no information to aid in interpreting ecosystem level effects, but was capable of displaying sensitivity equal to that of the SAM procedure and required a comparatively small time investment. We believe a useful new microcosm protocol could be developed by incorporating some of the strengths of the the SAM system into a mixed flask type system.

Keywords: Microcosms, Toxicity Testing, Copper, Ecosystem-level Effects, Testing Effort, Mixed Flask Culture, Standardized Aquatic Microcosm, Community Production, Community Respiration

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INTRODUCTION

Increasing interest in the use of microecosystems in aquatic toxicology has resulted in publication of several major works in recent years. Giesy's symposium volume [1] includes a variety of studies where specific ecological problems were investigated using microcosm systems. The National Academy of Sciences, National Research Council [2] at the request of the Environmental Protection Agency (Office of Toxic Substances), published a review of methods for ecotoxicology entitled "Testing for Effects of Chemicals on Ecosystems" to "identify characteristics of ecological systems that would indicate hazardous effects of chemicals beyond the level of single species." At about the same time, Oak Ridge National Laboratory, Environmental Sciences Division [3] issued a report entitled "Methods for Ecological Toxicology," which surveyed published reports of laboratory methods for measuring ecological effects and suggested tests for use in "predicting the effects of chemical substances on interspecific interactions and ecosystem properties."

As recent articles suggest, however, there remains a controversy over the relevance and adequacy of microecosystems in estimating environmental hazard. Cairns [4] makes a strong case for expanding the use and development of toxicity tests at organizational levels above that of a single species. Levin, et al. [5] support this view, citing the need to study toxic effects at the ecosystem level, and noting the utility of microcosms as one tool for such evaluations. Smies [6], on the other hand, argues that even though microecosystems may provide information that is more transferable to natural systems, problems with reliability and generality preclude their use in routine toxicity testing.

Despite these disagreements, it is generally conceded that microcosms do indeed have a valid role in toxicity testing. As Cairns [4] points out,

community and ecosystem-level toxicity testing is in a stage of development similar to that of single species testing in the 1950's and 1960's, when there was serious debate as to whether biological testing had any role in pollution assessment. Microcosm-based hazard assessments are appearing in the literature in increasing numbers, and several protocols for the use of microcosms in toxicity testing have been drafted. This proliferation of methods leads to a need for their systematic evaluation and comparison. Our broad objective, therefore, was to contribute to this process by comparing two approaches to the construction and use of laboratory aquatic microecosystems for hazard evaluation. These approaches, which we have chosen to call mixed flask cultures (MFC) and standardized aquatic microcosms (SAM), were developed respectively by Dr. John Leffler of Ferrum College, Ferrum, VA, and Dr. Frieda Taub and associates at the University of Washington, Seattle. Full copies of the MFC and SAM draft protocols are available from the Environmental Protection Agency and from the Food and Drug Administration, respectively.

The SAM protocol [7] calls for the development of a defined community from pure stock cultures of algae and small aquatic animals. The species assemblage does not simulate a specific community, but serves as a generalized model aquatic ecosystem which includes species at the primary and secondary trophic levels as well as decomposers. Changes in population densities, nutrient cycling and ecosystem level variables are monitored to evaluate toxic effects. This method provides a wealth of data but has been criticized for its costs. In addition Hammons [3] expressed concern that artificial communities may not be representative of "natural, co-adapted species assemblages," and suggested that they may not be reliable for studies of ecosystem level properties.

The MFC approach developed by Leffler [8] attempts to avoid some of these questions by using microcosms containing a community of organisms allowed to develop from a variety of natural sources over a period of time; a so-called "co-adapted" species assemblage. Evaluating toxic effects involves monitoring only ecosystem level changes (e.g., community production and respiration). Species population dynamics are ignored on the assumption that system level variables are species-independent. The method is considerably less labor intensive and not nearly so "data rich" as the SAM procedure. Critics have suggested, however, that the data collected are insufficient to provide a complete picture of toxic effects, and the variable communities which develop may result in problems with repeatability.

Our work with both types of microcosms has provided an opportunity to compare them in tests of the same toxicant. Specifically, we were interested in determining each system's sensitivity to copper and the repeatability of that response. In addition, our experience provided a basis for evaluating the ease of implementing the protocol, and the relative labor costs of the two protocols.

METHODS

Using the techniques described by Taub and Read [7] and Leffler [8], we ran tests to evaluate the response of SAM and MFC aquatic microecosystems to toxicant exposure. The SAM tests were run during our participation in an interlaboratory test of Dr. Taub's SAM protocol. Dr. Taub selected copper sulfate as the toxicant to be used in these tests, and, on the basis of previous experience, set exposure levels to yield measurable results in all treatments. To facilitate comparisons, we used a

similar range of copper sulfate concentrations, with the addition of several lower levels, in tests of the MFC protocol.

In running these experiments, the original protocols were followed as closely as possible. Although one of us (Harrass) had prior experience with the SAM protocol, the tests were implemented according to their written instructions. The tasks of microcosm set-up and sampling were done by initially inexperienced personnel at least some of the time during our tests so that time requirements were somewhat greater than those needed by experienced personnel. Two independent tests were completed with each system so that reproducibility as well as sensitivity could be evaluated.

SAM Protocol

For each experiment with the standardized aquatic microcosm (SAM) protocol [7], 30 microcosms were initially established. These were subsequently culled to a set of 24 which were used in the test. The treatment design consisted of a control and three treatment groups each containing six replicates, which were maintained in a 20 ^{o}C constant temperature room with illumination provided by a bank of fluorescent lights set to a twelve hour light-dark cycle.To ensure that all microcosms were illuminated equally, they were arranged in a randomized block design on a light intensity isophote beneath the light bank.

The microcosms were set up in acid washed 3.8-litre glass jars, each of which received a sediment of washed sand, chitin and cellulose and 3 litres of T82MV medium [7]. The filled jars were then sterilized by autoclaving. Upon cooling each jar was supplemented with filter-sterilized vitamins and other heat labile media components, and adjusted to a final pH of 7.0 with HCl. The mouths of all jars were kept covered to prevent evaporation and to minimize the possibility of airborne contamination.

Initially, microcosms were inoculated with each of ten algal species in the genera Anabaena, Ankistrodesmus, Chlamydomonas, Chlorella, Lyngbya, Nitzschia, Scenedesmus, Selenastrum, Stigeoclonium, and Ulothrix to a final concentration of 10^3 cells per ml. This algal community was then allowed a four day period to grow and develop in the absence of grazers. On day four, known densities of the cladoceran Daphnia magna, the amphipod Hyalella azteca, the ostracod Cypridopsis vidua, rotifers of the genus Philodina, and Hypotrich protozoans were added to the microcosms.

These systems were permitted to develop until day seven, at which time jars were assigned to treatment groups and dosed with copper(II) sulfate at rates of 0 (control), 500, 1000, and 2000 micrograms per litre Cu. Samples were collected from each jar immediately after treatment, preserved in 0.1N HNO_3 and analyzed for copper. Analyses were conducted by graphite furnace atomization techniques with a Perkin-Elmer Zeeman 5000 Atomic Absorbtion Spectrophotometer.

For the duration of the 63-day experiment, microcosms were monitored every Tuesday and Friday. On each of these sampling days, counts of population densities were made for all species. Additional information on primary productivity was collected by measuring in vivo fluorescence and optical density. Ecosystem level responses were followed by measuring changes in "pre-dawn" pH with a Beckman pH meter, and in dissolved oxygen concentrations with a Beckman 0260 Dissolved Oxygen Meter. By taking a three point dissolved oxygen measurement (evening, morning and evening) [9] around each sampling date, estimates of net daytime production (oxygen gain) and of night respiration (oxygen loss) were calculated. Samples of the medium were collected, filtered through a .45-micron membrane filter, and frozen twice a week for the first four weeks of the test and once a week thereafter. These samples were then analyzed for phosphate, ammonia

and nitrate using standard autoanalyzer techniques [10].

To simulate recruitment which might occur in a natural ecosystem, all microcosms were reinoculated on a weekly basis with algae and any missing animal species.

MFC Protocol

Mixed flask culture microcosms (MFC) were developed according to the protocol described by Leffler [8]. The microcosms were constructed in acid-washed one-litre beakers filled with 950 ml of Leffler's modified Taub #36 medium [8]. No sediment was used. Neither the containers nor the medium were sterilized since the inoculum was a mixed assemblage already containing many unknown species of microorganisms. The inoculum consisted of 50 ml. of stock taken from a community of mixed algae, zooplankton and other microorganisms which had been allowed to develop and mature for at least three months in laboratory aquaria filled with Taub # 36 nutrient medium. In accordance with the protocol, no effort was made to quantify the numbers or determine the species of organisms being added. Instead the stock was simply examined microscopically to verify the presence of specified minimum functional groups: two species of edible, unicellular green algae, one species of nitrogen fixing blue-green alga, one species of filamentous green alga, one species of herbiverous grazer, one species of benthic detritivore, bacteria and protozoans. In contrast to the SAM protocol, the MFC containers were left uncovered, so that evaporative loss would provide sufficient free volume for weekly re-inoculation. If evaporative loss was excessive, microcosms were brought up to volume each week with distilled water.

The MFC technique prescribed a somewhat different schedule of development and testing than the SAM procedure. The pre-dose development

period was much longer, requiring six weeks rather than one week, while the exposure period was shorter, lasting 42 days in contrast to the 56 days in the SAM protocol. Part of the reason for the longer development time is the requirement for an initial two week period of cross seeding designed to effect a more uniform distribution of organisms among the beakers. This procedure required that, on a semi-weekly basis, aliquots be withdrawn from all microcosms, mixed and re-distributed. Additionally the longer development time is believed to allow the systems to reach something resembling a steady state by the time of toxicant addition. As in the SAM protocol, all microcosms were reinoculated on a weekly schedule using a 50 ml quantity of mixed aquarium stock culture.

The microcosms were arranged in a randomized block design in a Percival I-60 Environmental Growth Chamber at 20 ^{o}C. Fluorescent light banks provided illumination on a twelve hour light-dark cycle. To guard against potential effects from non-uniform incubator conditions, all blocks were rotated to a different shelf position twice weekly. In the first experiment a total of 35 microcosms were started and followed through the pre-dose phase. Prior to dosing, five "outliers" were culled from the set, so that the actual treatment design involved 30 microcosms divided into a control and five treatment groups each containing five replicates. These groups were dosed to copper concentrations of 0(control), 36, 70, 363, 725, and 1450 micrograms per litre respectively. Due to the availability of additional incubator space, 40 microcosms were started and an additional treatment group was used to permit copper dosages of 0, 70, 320, 500, 750, 1450 and 2000 micrograms per litre in the second run.

Pre-dose monitoring was done on a weekly schedule, although the protocol only requires one set of pre-dose measurements taken two days

prior to toxicant addition. After treatment, monitoring was done on four consecutive days the first week, two days the next week and once weekly thereafter. Only ecosystem level variables were measured. As in the SAM systems a three point dissolved oxygen determination (using an Orbisphere 2607 Dissolved Oxygen Meter) was used to estimate net daytime production (oxygen gain) and night respiration (oxygen loss). Both "pre-dawn" and late afternoon measurements of both pH and Eh were also taken using a Fisher 525 meter equipped attached to a junction box with standard pH electrodes and a platinum Eh electrode. Since Eh values are highly dependent on pH they were subsequently standardized to pH 7 using the formula E7(mv) = Eh(mv)+ 58(pH-7) [11]. No counts of population densities were made, although gross visual observations of change in algal or zooplankton abundance were recorded. A summary of the experimental design for both the SAM and MFC protocols is shown in Table 1.

Protocol Comparisons

The data collected in these experiments were used to evaluate the relative sensitivities of these two systems and to compare the repeatability and consistency of the results obtained with each. The minimum significant effect concentrations were determined by running an ANOVA to determine whether there was a significant difference among groups. In cases where differences were found, Dunnett's procedure [12] was used to determine which treatments differed from the control. Sensitivity was evaluated on the basis of the overall frequency with which each level of copper treatment caused significant differences from control variables. Repeatability was judged both on the similarity of control group development between runs of a test system and the consistency with which copper toxicity levels were determined. A final point of comparison between

these two systems involved an attempt to balance the amount and utility of information gained against time and labor required.

RESULTS and DISCUSSION

The Media

The media used in these protocols were similar nutrient rich formulations, although they differed in several potentially important aspects. The T82MV medium used in the SAM tests contained 7.00 mg. per litre NO_3-N and 1.23 mg. per litre PO_4-P. The modified Taub #36 medium used in the MFC tests contained less than one third as much nitrogen (2.12 mg. per litre NO_3-N), but contained more than twice as much phosphorus (3.10 mg. per litre PO_4-P). These are all extremely high nutrient levels in comparison with natural waters where N and P levels are typically measured in terms of micrograms per litre. They are not, however atypical of the nutrient levels often found in microcosm or algal growth media.

The SAM medium further differed from the MFC in that it contained added vitamins, while the MFC medium did not. Both media contained EDTA as a chelator to prevent precipitation of iron and other trace metals from the medium. The presence of EDTA represented a potential problem for evaluating copper toxicity. As a chelating agent, EDTA might be expected to remove a portion of the toxicant from solution. The amounts of copper actually chelated in these systems was apparently quite low, however. At most, the 1.42 micro-molar concentration of EDTA in T82MV could theoretically chelate a maximum of 1.42 micro-moles or 90 micrograms per litre of copper. In tests with specific ion electrodes, Taub *et al* [13] found no difference in cupric ion activity when copper was added to media containing no EDTA and to T82MV media. This is possibly a result of EDTA chelating capacity having

already been occupied with other ions, since the amount used is approximately equivalent to the combined molality of iron and trace metals.

The EDTA concentration in the MFC systems was an order of magnitude higher at 11.2 micro-molar. This level of EDTA could theoretically complex up to 712 micrograms per litre copper, which would result in a drastic reduction of copper levels in the treated microcosms. It appears, however, that this additional EDTA was fully complexed with the equivalently higher iron and trace metals, or that the long six week development period allowed biodegradation of EDTA, since we were able to observe copper effects at levels down to 36 micrograms per litre. We conclude then that copper complexing by EDTA was not a significant problem in either of these systems.

Effects of Copper

Copper Levels

We view both of these microcosms as functioning systems with many of the properties of natural ecosystems. Their overall response to a toxicant is the result of interactions among many biotic and abiotic factors, rather than sum of individual species effects. Thus when a toxicant is added to a microcosm test system, we are not treating the organisms within the microcosm with a certain level of toxicant as much as we are dosing the entire ecosystem. In this respect then, microcosm tests are quite different from single species exposure tests. Once a toxicant is added to a microcosm, it is quickly partitioned among the biota, the liquid phase, and other abiotic components of the system. A measurement of toxicant in the liquid phase, unless taken immediately after addition, is of little value in determining the amount of toxicant in the system. For this reason we did

not routinely measure aqueous copper levels in the test systems. The same analyzed copper stock solution was used to dose all microcosms in all experiments, so we are confident the systems actually received the indicated doses. The SAM protocol specified that samples be taken immediately after copper was added on day 7. Analysis of these samples indicated that copper levels in SAM # 1 and SAM # 2 were an average of 3% and 6%, respectively, above nominal concentrations.

Ecosystem level variables in common

Oxygen-related variables

During the first few days of exposure all microcosms generally responded to copper in a similar fashion. There was an initial decline in oxygen gain (Fig. 1) and oxygen loss at all levels of copper treatment in the SAM systems and at levels down to 320 micrograms per litre in the MFC systems. The size and duration of these effects were dose-related with both systems, and were of consistently greater magnitude in the SAM microcosms.

This dose-response relationship was verified by regressing oxygen gain during the first sampling date after treatment (day 43 in the MFC protocol and day 11 in the SAM protocol) against the log of [Cu++] + 1.0 (Table 2). Calculated F values indicated that all regression coefficients (b) were significantly different from zero at $p <= .01$. A comparison of the slopes of these lines using an analysis of covariance [14] showed no significant difference among slopes ($F= .833$; $p <= .001$).

Following this initial inhibition, measurements of oxygen gain and loss began to recover in all systems, with some response patterns becoming non-monotonic (i.e. responses at low treatment levels were not always intermediate to responses shown at higher and control levels) for a time. The lowest treatment level in the SAM experiment (500 micrograms per litre

copper) recovered rapidly in both runs, so that by day 21, oxygen-related variables were significantly greater than controls. At higher copper levels these measurements recovered slightly, but remained below control values throughout the SAM experiments. In the MFC experiments the lowest treatment levels (36 to 70 micrograms per litre) had no statistically significant effects on oxygen measurements. Intermediate levels (320,360 micrograms per litre) showed recovery to control levels within a week, while higher concentrations demonstrated prolonged periods of reduced oxygen gain and loss.

pH-related variables

Pre-dawn pH values (Fig. 2) varied in a manner similar to the pre-dawn dissolved oxygen measurements; an expected result in view of the relationship between dissolved oxygen, carbon dioxide and pH. Since these measurements were taken before lights on they were primarily a function of night respiration. Respiration associated increases in carbon dioxide levels result in decreased pH. Therefore pH values lower than control levels may be indicative of increased respiratory activity.

As noted with the oxygen measurements, the magnitude of pH change associated with copper treatment was considerably less with the MFC systems than with the SAM systems. While differences of nearly three pH units were observed among SAM groups (Fig. 2a, c) corresponding MFC systems showed shifts rarely exceeding one pH unit (Fig. 2b, d). In general, however, the pH data from each system showed a similar pattern. After a sharp initial pH drop in all treatment groups, values rose toward control levels.

In the SAM experiment all treatments attained levels significantly above the control from days 10 through 35, although the response patterns

were again non-monotonic. Recovery after day 35 occurred in a concentration-related pattern. The low copper group returned to control levels by day 39, followed by the 100 micrograms per litre group on day 53. Values in the high copper group remained elevated through the end of both tests. With the MFC systems, pH proved to be a more sensitive measure of effect than oxygen. All treatments between 36 micrograms per litre and 725 micrograms per litre Cu caused statistically significant increases in pH in the first test, but not in the second. These higher values were first observed 14 days after treatment and usually persisted until the end of the test.

Additional variables

In the MFC protocol Eh was the only additional variable measured. As a measure of redox potential Eh can be affected by a variety of ion species. In the case of aquatic microcosms, with their abundant algal communities however, Eh (when corrected for pH) is most directly correlated with oxygen. As a result, the observed patterns of Eh change were very similar to the dissolved oxygen figures and provided little in the way of new information.

The multitude of additional measurements in the SAM procedure provided a large body of supplementary data. With these data we could develop a more complete picture of events associated with the observed system level changes. As an example, Fig. 4 shows, in summary fashion, a number of variables measured for the 500 micrograms per litre Cu treatment group. The increase and subsequent decline in primary production in this group inferred from the oxygen gain measurements can clearly be ascribed to changes in the algal community documented from other observations. Fluorometry values (a measure of in vivo chlorophyll) showed a very close

correlation with the production curve. Similarly, the peak in algal biovolume coincided well with the observed peak in production. More specific data on algal population densities showed the increase in algal biovolume to be almost entirely the result of a bloom in Ankistrodesmus sp. The additional SAM data also provided an explanation of the decline in production which occurred toward the end of the test: as the algae were increasing in number, nitrate levels were declining steadily to levels near zero, forcing nutrient competition, and inhibiting further population growth. Furthermore, in response to an increased food supply and presumably reduced levels of active copper, the density of Daphnia magna began to increase rapidly. Ultimately their grazing activities reduced the algal community to very low densities. In the face of this reduced food supply the Daphnia again declined to densities well below their peak. It is the integrated effect of all these changes then, that is reflected at the ecosystem level.

Comparative System Sensitivities

Sensitivity of test ecosystems, defined as the ability to detect responses to stress, is a function of both the test system and the experimental design. Test systems which do not include organisms sensitive to the toxicant, or include some factor which affects exposure to the toxicant, are expected to be less sensitive than test systems without these properties. Similarly, experimental designs which depend on a small number of replicates or allow testing of only a few toxicant concentrations have a reduced statistical ability to detect effects and accurately determine minimum effect levels.

Theoretical Sensitivity

The experimental design and information about variability among

replicate test microcosms is useful to estimate the theoretical ability to detect statistically significant differences. Conquest [15] showed how the coefficient of variation (CV, the ratio of the sample standard deviation to the sample mean) may be used to estimate a Minimum Dectectable Difference (MDD) between control and treatment means for a given experimental design. The MDD defines the proportion by which treatments must differ from controls to demonstrate a significant difference, and thus may be viewed as an estimate of the statistical "resolving power" of a protocol.

Data from the control groups were used to calculate the mean CV for several of the parameters observed in both systems during the post-treatment period (Table 3). In general, the CV's were lower for the MFC data than for the SAM data. For dissolved oxygen data, SAM control group CV's were 2-3 times those of tne MFC control groups. For pH data, only slight differences were noted. This greater variability in the SAM systems was at least in part due to differences in the timing of events among treatment group replicates (i.e. shifts in community structure were slightly out of phase among replicates). MDD values calculated using the estimated CVs and information from the experimental designs, suggested that MFC tests would require a smaller shift in treatment mean values to demonstrate significant effects and hence would be considered more sensitive.

For the MFC protocol, with an estimated CV of 2.34% for pH data (from both runs), the estimated MDD is 0.0497, i.e., a 5.0% difference between control and treatment group means would usually lead to a statistically significant difference. For the dissolved oxygen data, using 5.22% as the estimated CV, the estimated MDD is .1110.

For the SAM protocol, with an estimated CV of 2.98% for pH data, the

estimated MDD is 0.0638. For dissolved oxygen levels, using an estimated CV of 13.95%, the estimated MDD is .3069. (These are calculated based on probability of type I error (alpha) of 0.05, and type II error (beta) of 0.10.).

The observed coefficients of variation thus indicate that the MFC protocol has generally demonstrated less variability for those measurements made in common, and so should have greater sensitivity (i.e. be able to statistically detect smaller changes associated with treatment.) Note, however, that the CV values, and consequently the MDD values, varied substantially between the first and second runs of each protocol. For the MFC experiments, the CV's of the first run were less than 50% of the CV's for dissolved oxygen data on the second run. Also, the CV's of the second MFC experiment were only slightly less than the CV's of the first SAM experiment, indicating that the real differences in inherent variability of measurements under the two protocols may not be great.

The MDD's of the extra variables measured in the SAM protocol were generally higher than those of ecosystem level measurements, suggesting these would be less sensitive measures of effect. This has been noted by Harrass and Taub [16], who observed that population densities typically range over several orders of magnitude, causing CV's to vary greatly during an experiment.

Observed Sensitivity

The determination of a minimum effects level and of a maximum no-effect level would be the optimal approach to comparing test ecosystems' actual sensitivity to a toxicant. All copper concentrations used in the SAM experiments (set as part of an interlaboratory comparison of the protocol) demonstrated significant effects; consequently a maximum no-effect level

could not be identified. Similarly concentrations used in the MFC tests were all above no-effect levels. The 320 micrograms per litre treatment was the minimum copper concentration causing a system level inhibitory effect. At 36 and 70 micrograms per litre Cu, pH measurements were significantly elevated above the controls in MFC # 1, presumably a result of grazers being selectively killed.

As an alternative method of comparing the sensitivity of actual observations in identifying treatment effects, we compared the frequency of observed significant differences between treatment groups and control. Using this approach, more sensitive systems would show a higher frequency of observed significant differences over the course of an experiment, reflecting major and prolonged effects.

A priori comparisons were made between the control microcosms and microcosms of each treatment group (t-tests) for each day of observation ($p<=.05$). For each experiment the percent frequency of observed significant differences was calculated for oxygen- and pH-related variables. Since observations are made on different schedules in the two protocols, only measurements taken at comparable times after treatment were used in this analysis. For each treatment level, the highest percent frequency calculated from any variable was plotted against copper concentration in Fig. 4. This approach, while obscuring the time-dependent patterns of the data, provides an indication of the extent to which each treatment level was detectably different from controls.

From this analysis of sensitivity (based only on those ecosystem variables measured in common) we conclude that the first MFC experiment and both SAM experiments were equally sensitive, but the second MFC experiment was clearly less sensitive. Again, this analysis is limited because no data were obtained from SAM tests at treatment levels comparable to the low

levels used in the MFC experiments.

The basic dose-response patterns are similar in all tests; higher treatment levels produced greater frequencies of significant effects. Inconsistent dose-response relationships, especially at lower copper concentrations, were due to time-related shifts from inhibitory to compensatory or stimulatory patterns.

The similarity between the two SAM runs is quite clear. Conversely, the two MFC runs were markedly different. In the first MFC run, at 725 micrograms per litre treatment, up to 100% of the observations were significantly different from controls, while in the second run only up to 38% of the observations were different.

We used the same analysis of maximum frequency of observed significant differences to determine whether the additional variables monitored in the protocols were equally sensitive to treatments. In the MFC protocol, Eh was the only additional variable, and was only occasionally found to be more sensitive. In the SAM protocol, the additional measurements of nutrient levels, in vivo fluorescence, optical density, and population densities were generally less sensitive than the oxygen and pH measurements. Again, this analysis must be limited by the lack of SAM data from application levels showing moderate effects. These observations are consistent with the higher MDD values noted previously for these variables.

Protocol Design and Sensitivity

We have presented somewhat conflicting conclusions about the statistical properties of the two protocols. The MFC protocol appears to have better theoretical capability to demonstrate treatment effects because of its lower variability and MDD. However, the SAM protocol actually did better in consistently demonstrating treatment effects at equivalent

application levels.

Differences in the protocols suggest a reasonable explanation for this pattern: the MFC protocol uses a 6 week development time in order to attain a relative maturity, while the SAM protocol allows only 1 week before treatment. The cross-inoculation and long development time thus appear to be successful in attaining stability and similarity of microcosms within a given experiment, as reflected in the comparatively low CV observed in the MFC controls. The SAM protocol is designed so that treatments occur during the period of greatest development and change; consequently the magnitude of effects is greater than would be observed in aged, stable systems. The effects of nutrient stimulation in the SAM systems at different times (new vs. aged) were studied by Kindig [17], who observed that SAM responses were of greater magnitude and detectability when treatment occurred early in microcosm development.

Repeatability and Consistency

For toxicological data, constancy of test conditions is of major importance. Field conditions usually preclude this for ecological data, which provides a major rationale for test ecosystem protocols. To be useful, test system protocols must demonstrate high degrees of repeatability (i.e. the data obtained from independent experiments must lead to the same conclusions).

The two runs with each protocol allowed us to evaluate repeatability and consistency of response. In general the effects in the second experiments were similar to those observed in the first, although some differences were apparent.

In the SAM tests the sequence and timing of events were remarkably similar. All groups showed an initial decline in oxygen gain (Fig. 1)

followed by a recovery of variable magnitude. As in the first test, the 1000 and 2000 micrograms per litre Cu groups in the second test were held at significantly lower levels of production and respiration for the duration of the test. The 500 micrograms per litre Cu group showed similar periods of increased oxygen gain, peaking on day 28. The variability among replicates was considerably less than in the first test.

In contrast, variability among replicates was increased in the second MFC test, making it more difficult to identify statistically significant differences. Although inhibited oxygen gain and loss were again observed, the duration of these effects was considerably less, as the systems displayed more resiliency in the second test. The decline in oxygen gain (Fig. 1), for example, persisted only 18 days even at 2000 micrograms per litre Cu, in contrast to the first test where production was inhibited by 725 micrograms per litre Cu for the duration of the test.

To evaluate the constancy of test conditions, data from pretreatment observations and from control systems can be compared. Using oxygen gain as an indicator, the development of the control groups from all four tests was plotted against time (Fig. 5). Clear stages of increasing and decreasing oxygen gain, reflective of cycles in algal and grazer population densities, were apparent in the SAM systems. Similar maturational sequences appeared in the MFC systems. The SAM controls showed quite similar levels of oxygen gain in both tests. The MFC controls developed quite differently in each test, however. The much higher oxygen gain values recorded in the second MFC controls were consistent with visual observations which indicated the development of a very dense algal community, and lower zooplankton densities. This more dense algal community presumably removed copper from solution at a greater rate, accounting for the observed decrease in system

sensitivity.

Generality

Although the major thrust of this work was to compare two protocols and not to evaluate how well microcosm results agree with either single species or field results, it is of interest to note how the toxicity data obtained in these experiments compare with those of other investigators.

Copper toxicity is determined by many factors. The hardness, concentrations of total organic carbon (TOC), iron, and other copper complexing agents in water or culture media can all affect the amount of free copper in solution [18]. In addition, many algae are capable of excreting complexing substances in response to metal stress [19],[20]. These variables, in combination with the variety of test systems which have been used and the types of endpoints measured, make comparisons among results extremely difficult.

There are some general trends among the numerous copper toxicity data reported in the literature. Most studies have shown that aquatic plants are less sensitive to copper than animals. In a recent review [18] chronic copper toxicity values were summarized for a variety of organisms from a number of literature studies. Chronic values reported for zooplankton range from 10 [21] to 49 micrograms per litre [22]. While the range of copper values reported to inhibit algal growth is extremely large, varying from 1 [23] to 8000 micrograms per litre [24], most reports suggested minimum inhibitory concentrations of 100 micrograms per litre and above. On this basis, the effect levels the MFC tests fall within the range of reported single species results.

Other workers have found varying degrees of similarity between laboratory microecosystem and field data. Harrass and Taub [16] concluded

that the types of responses observed in SAM tests and reported from field tests were similar, although they found copper effects in SAM systems only at concentrations exceeding those usually considered toxic in natural communities. These differences were attributed to high productivity in the microcosms. Giddings [25] has suggested that, because of the reduced species numbers, microcosm tests may be more sensitive to stress than more diverse natural communities, where alternative species may replace impaired species. Hardy [26] has noted general similarities between SAM responses to streptomycin and malathion and effects observed in similarly-sized systems obtained from Lake Washington.

Relative Costs

The time requirements for the two protocols were markedly different. As expected, the SAM procedure was much more time intensive, requiring 425 hours per nine week test compared with only 67 hours per 13 week MFC test (Table 4). Most of the additional time is that required for monitoring population densities in the SAM systems, although nutrient analysis also adds significantly to the total. These values do not include the time for data analysis, which will vary widely depending on the degree of computerization of the data gathering and analysis process. Thus the labor investment is more than six times greater for the SAM protocol, than for the MFC method.

To some extent, this time differential could be reduced as the staff gained proficiency; for example, the estimated time required for monitoring populations in Dr. Taub's laboratory is approximately one half our estimate[1]. It is obvious however, that the costs of running SAM tests are considerably greater than the MFC system.

For this additional cost there is an increase in reproducibility and a wealth of data to help explain or interpret observed ecosystem level changes. For example, our hypothesis that the differences between runs of the MFC protocol were due to substantial differences in the algal biomass present could have been tested if more quantitative data about algal abundances had been obtained. Similarly, the oxygen and pH data often showed non-monotonic responses in these experiments. The SAM protocol has an advantage in such a situation, since the additional data can verify possible mechanisms about the nature of the observed responses.

The higher time requirement of the SAM procedure, which translates into about 1.25 full-time personnel equivalents for the duration of the test, suggests that this protocol is very expensive for routine use. Since we found that system-level variables were sensitive to treatment and were consistent with responses monitored by the other data collected, including population counts, it would be feasible to modify the test protocol such that only system-level variables were routinely monitored. Population and other data could be obtained to supplement and explain the routine data as appropriate.

SUMMARY

Repeated runs of two test ecosystem protocols showed generally similar changes in system level variables in response to copper sulfate treatment. No minimum effect level was determined for either system, but effects in the MFC test were seen at copper concentrations within the range of reported chronic single species data. The MFC systems showed less variability within control groups, and consequently had a better theoretical ability to detect effects. However, the SAM protocol called for chemical treatment during a highly sensitive stage of ecosystem

development, and so was able to demonstrate sensitivity equal to that of the MFC system at comparable treatment levels. The population densities and additional variables monitored as part of the SAM protocol provided useful explanations for the observed system level changes, but were not markedly better indicators of impact. An added advantage of the MFC system was its experimental design which allowed testing a greater number of toxicant concentrations.

The amount of time required to conduct the SAM protocol was substantially (6X) greater than needed for the MFC protocol. Monitoring of only system-level variables on a routine basis would substantially reduce the labor required for the SAM protocol, without reducing the ability to detect significant changes. Alternatively, better control of inoculation conditions, and supplemental monitoring of MFC functional group densities would enhance the ability of MFC tests to demonstrate repeatable responses. As experimental scientists, we prefer to have more data rather than less, in order to present more complete explanations. A routine hazard assessment protocol does not, however, have to provide complete explanations about why things happened. If a microcosm protocol is to be useful as a warning, some suggestions about mechanisms of effects may be needed to provide direction for further testing. By analogy, warning lights alone may be adequate in many cars, but gauges may still be essential in some situations. A preference for a detailed over a more general type of data (or vice versa) may depend on decisions and policies other than the overall cost of an ecosystem hazard assessment test. We feel then, that a relatively sensitive, repeatable microcosm test procedure could be developed by incorporating the strong points of the MFC and SAM procedures into a hybrid protocol.

Figure Captions

FIGURE 1. Differences in oxygen gain (production) between copper treatments and the control. A) SAM experiment # 1, B) MFC experiment # 1,C) SAM experiment # 2, D) MFC experiment # 2. Each point was determined by subtracting the control mean from the treatment group mean. RND = Range of non-significance as determined by Dunnett's test (alpha = .05). Points which lie outside the shaded area are significantly different ($p < .05$) from the control. Legend refers to copper (II) ion.

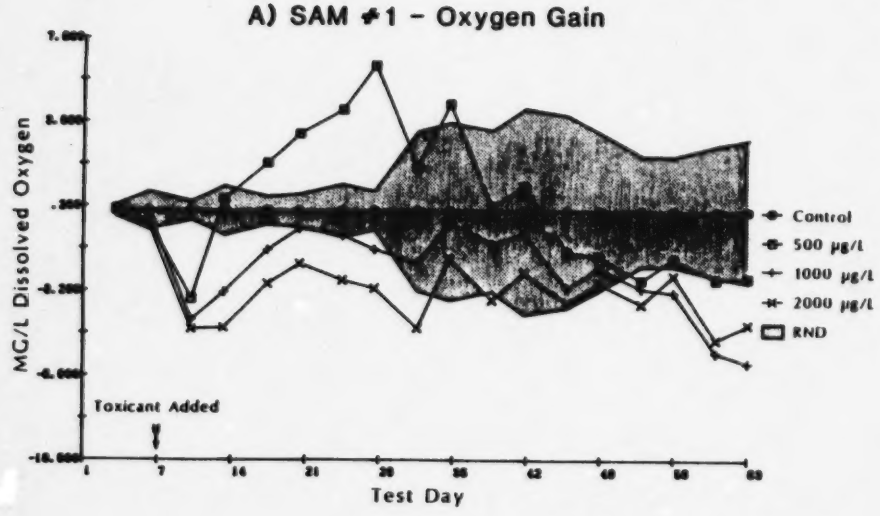
FIGURE 2. Differences in morning pH between copper treatments and the control. A) SAM experiment # 1, B) MFC experiment # 1,C) SAM experiment # 2, D) MFC experiment # 2. Each point was determined by subtracting the control mean from the treatment group mean. RND = Range of non-significance as determined by Dunnett's test (alpha = .05). Points which lie outside the shaded area are significantly different ($p < .05$) from the control. Legend refers to copper (II) ion.

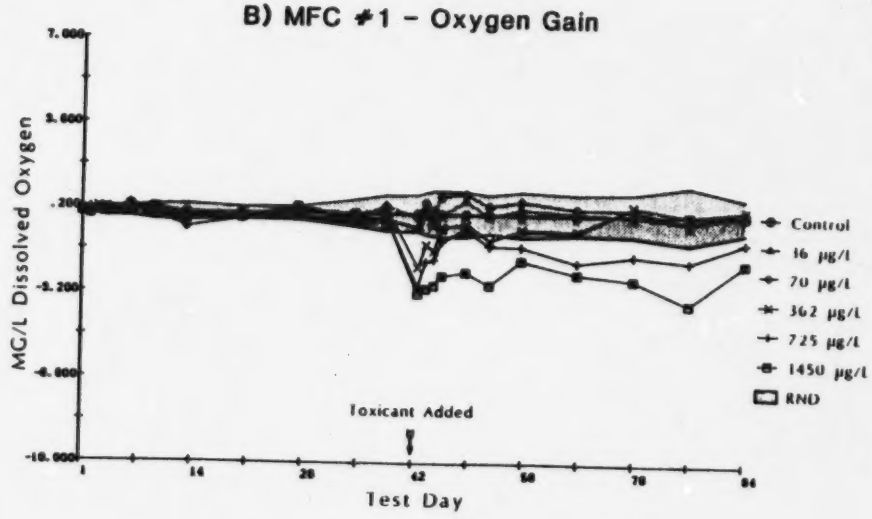
FIGURE 3. Additional biological and chemical variables in response to 500 micrograms per litre Cu, SAM experiment # 1. A) Production (oxygen gain) and Respiration (oxygen loss), B) *in vivo* Fluorescence, C) Algal Biovolume, D) *Ankistrodesmus*, E) Total *Daphnia*, F) Nitrate + Nitrite Nitrogen.

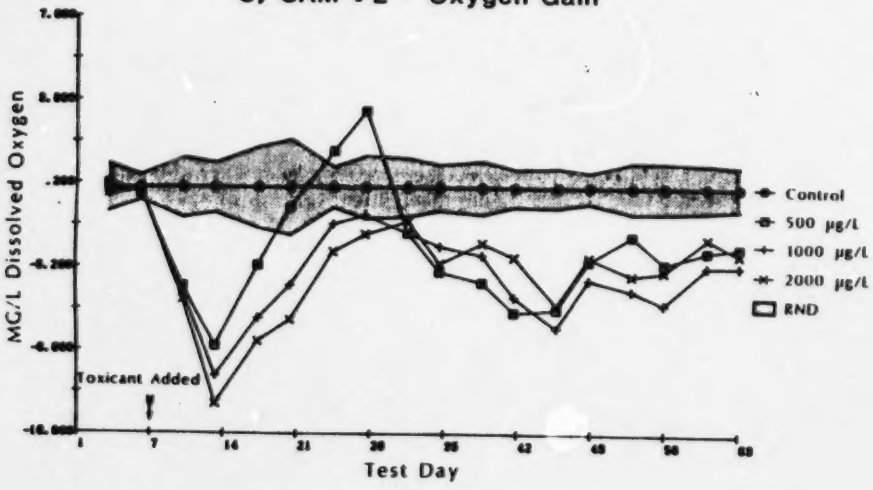
FIGURE 4. Maximum Frequency of Significant Differences Observed During Each Run of the SAM and MFC Protocols.

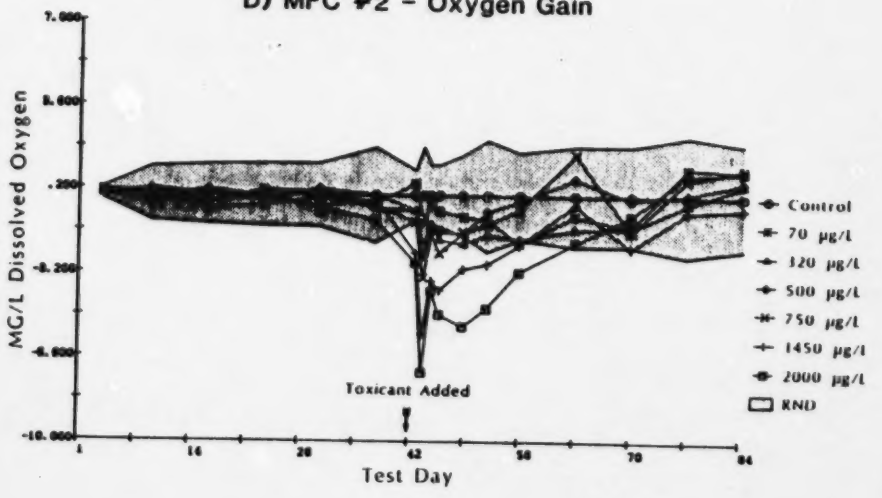
FIGURE 5. Production in control groups for both SAM and MFC experiments.

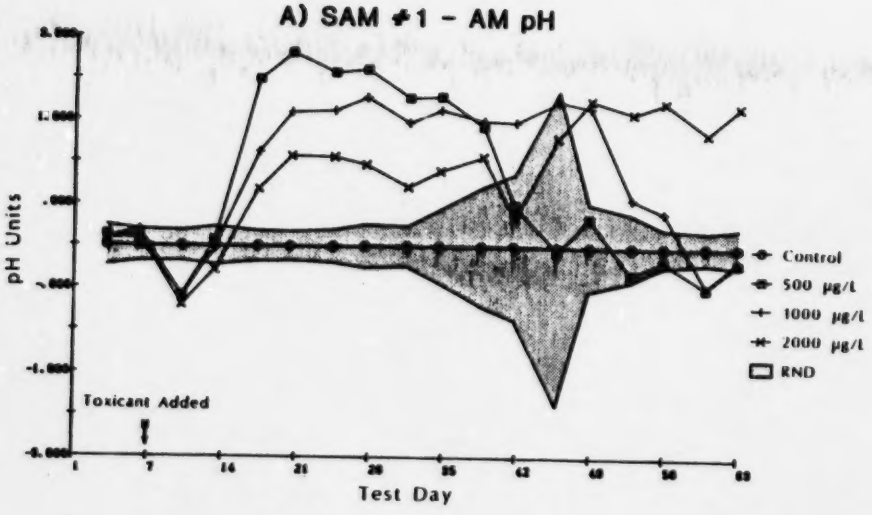
Fig. 1

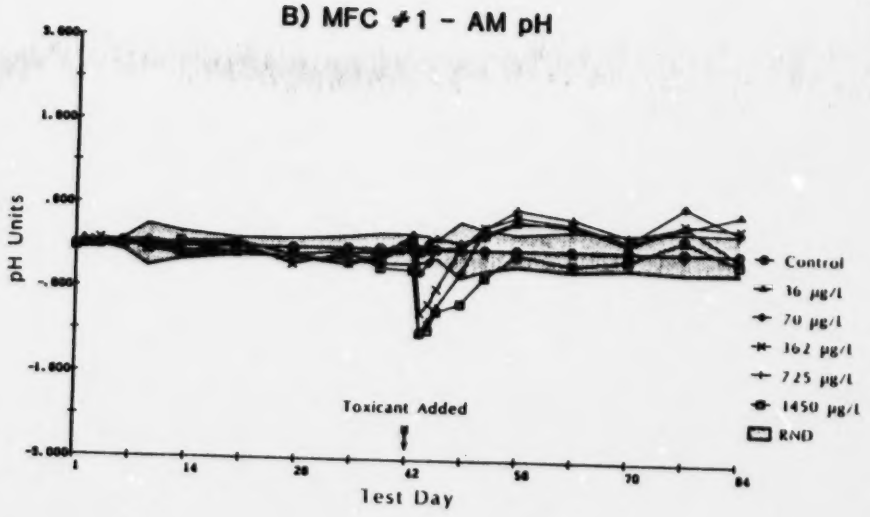

A) SAM #1 - Oxygen Gain
MG/L Dissolved Oxygen
Test Day
Toxicant Added
Control
500 µg/L
1000 µg/L
2000 µg/L
RND

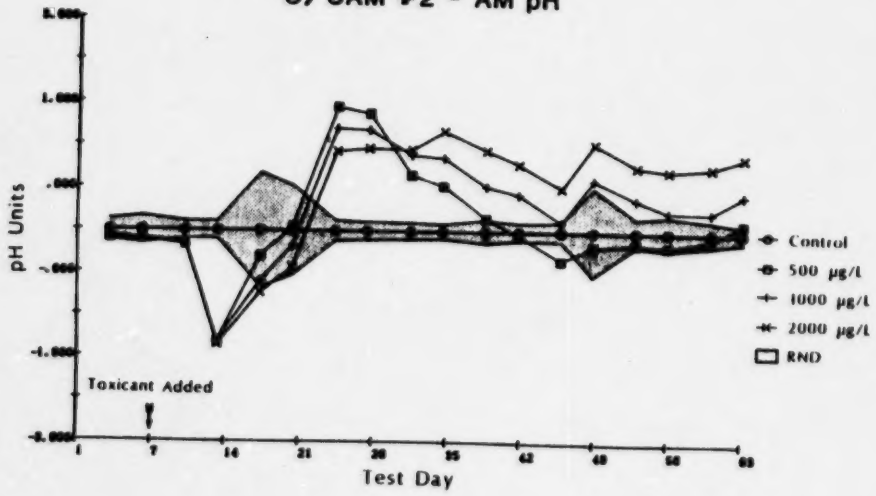

B) MFC #1 - Oxygen Gain
MG/L Dissolved Oxygen
Test Day
Toxicant Added
Control
16 µg/L
70 µg/L
362 µg/L
725 µg/L
1450 µg/L
RND

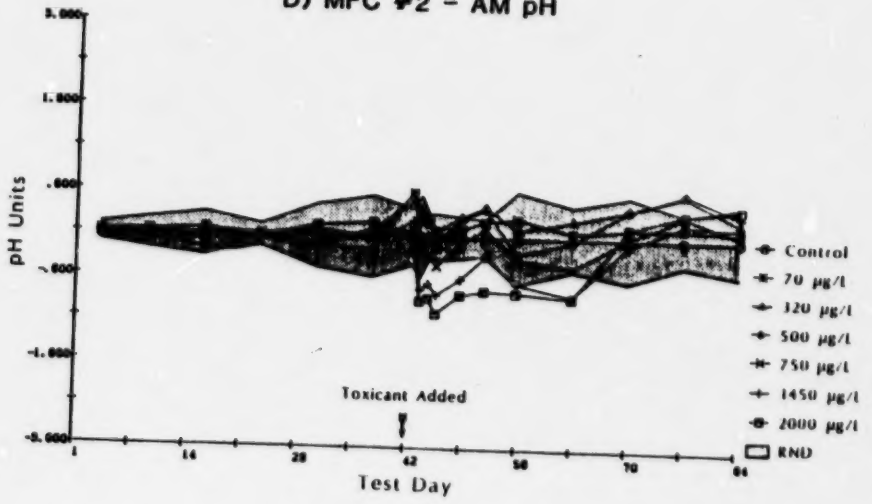

C) SAM #2 - Oxygen Gain
MG/L Dissolved Oxygen
Test Day
Toxicant Added
Control
500 µg/L
1000 µg/L
2000 µg/L
RND

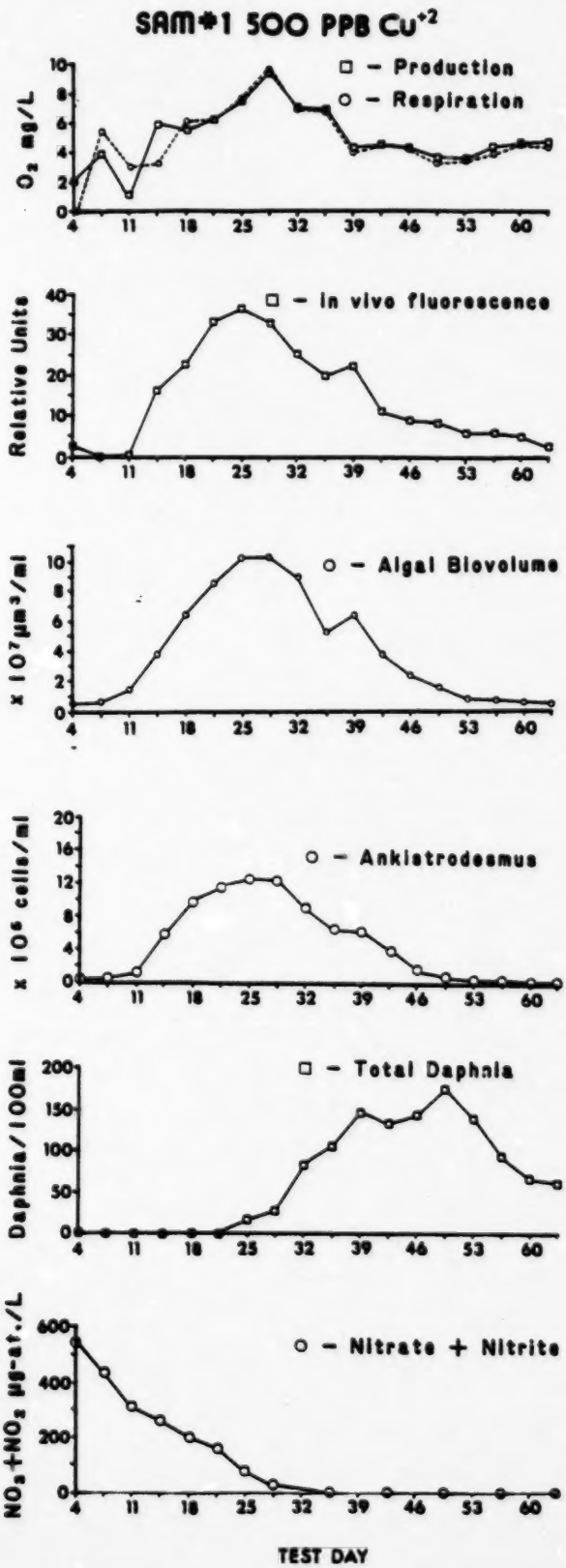

D) MFC #2 - Oxygen Gain
MG/L Dissolved Oxygen
Test Day
Toxicant Added
Control
70 µg/L
320 µg/L
500 µg/L
750 µg/L
1450 µg/L
2000 µg/L
RND


A) SAM #1 - AM pH
pH Units
Test Day
Toxicant Added
Control
500 µg/L
1000 µg/L
2000 µg/L
RND


B) MFC #1 - AM pH
pH Units
Test Day
Toxicant Added
Control
16 µg/L
70 µg/L
362 µg/L
725 µg/L
1450 µg/L
RND


C) SAM #2 - AM pH
pH Units
Test Day
Toxicant Added
Control
500 µg/L
1000 µg/L
2000 µg/L
RND


D) MFC #2 - AM pH
pH Units
Test Day
Toxicant Added
Control
70 µg/L
320 µg/L
500 µg/L
750 µg/L
1450 µg/L
2000 µg/L
RND


SAM*1 500 PPB Cu+2
□ – Production
○ – Respiration
O2 mg/L
□ – in vivo fluorescence
Relative Units
○ – Algal Biovolume
x 10⁷µm³/ml
○ – Ankistrodesmus
x 10⁶ cells/ml
□ – Total Daphnia
Daphnia/100ml
○ – Nitrate + Nitrite
NO3+NO2 µg-at./L
TEST DAY

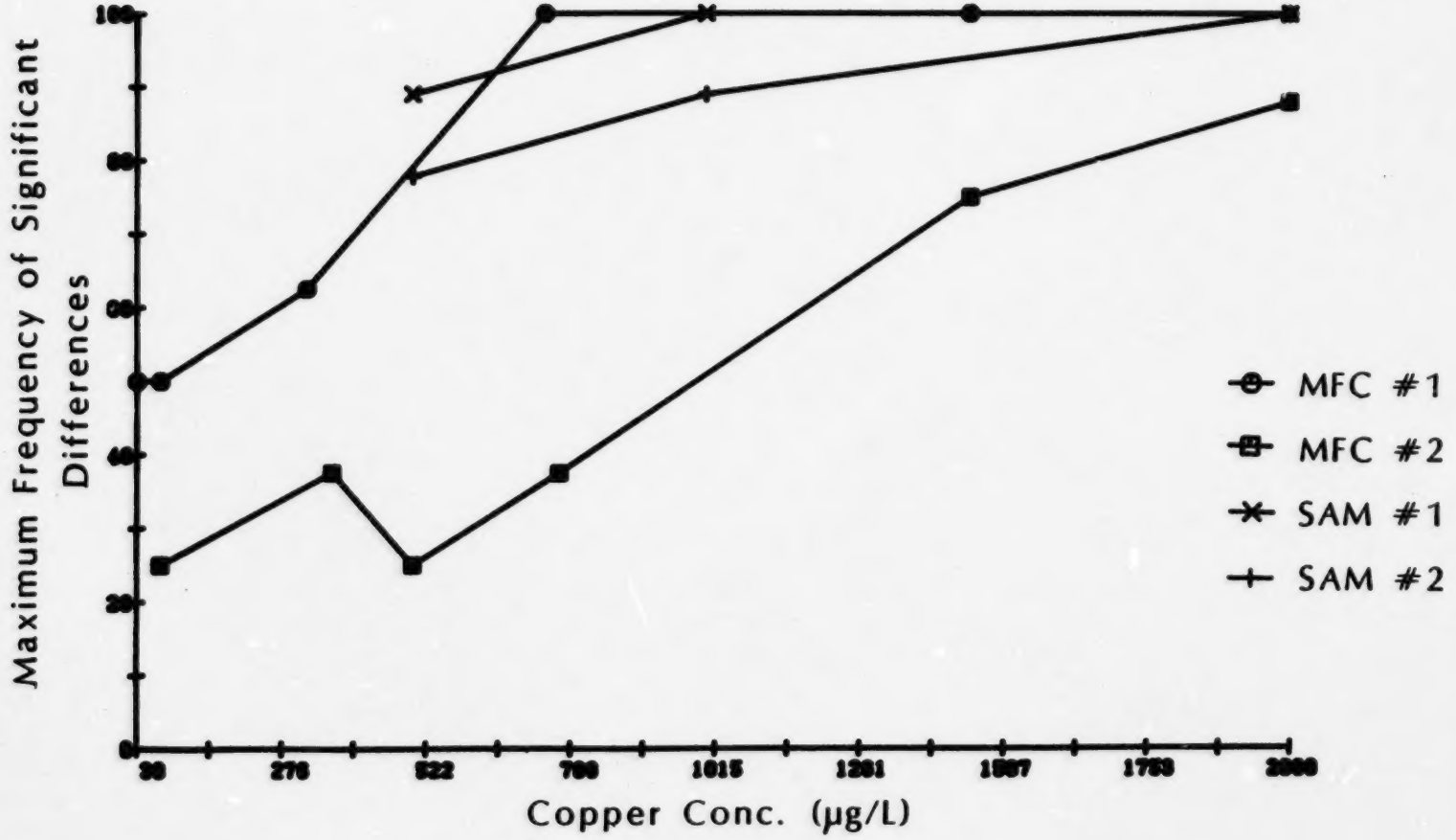

Maximum Frequency of Significant Differences
100
80
60
40
20
0
30
278
522
768
1015
1261
1507
1753
2000
Copper Conc. (µg/L)
MFC #1
MFC #2
SAM #1
SAM #2

Fig. 5

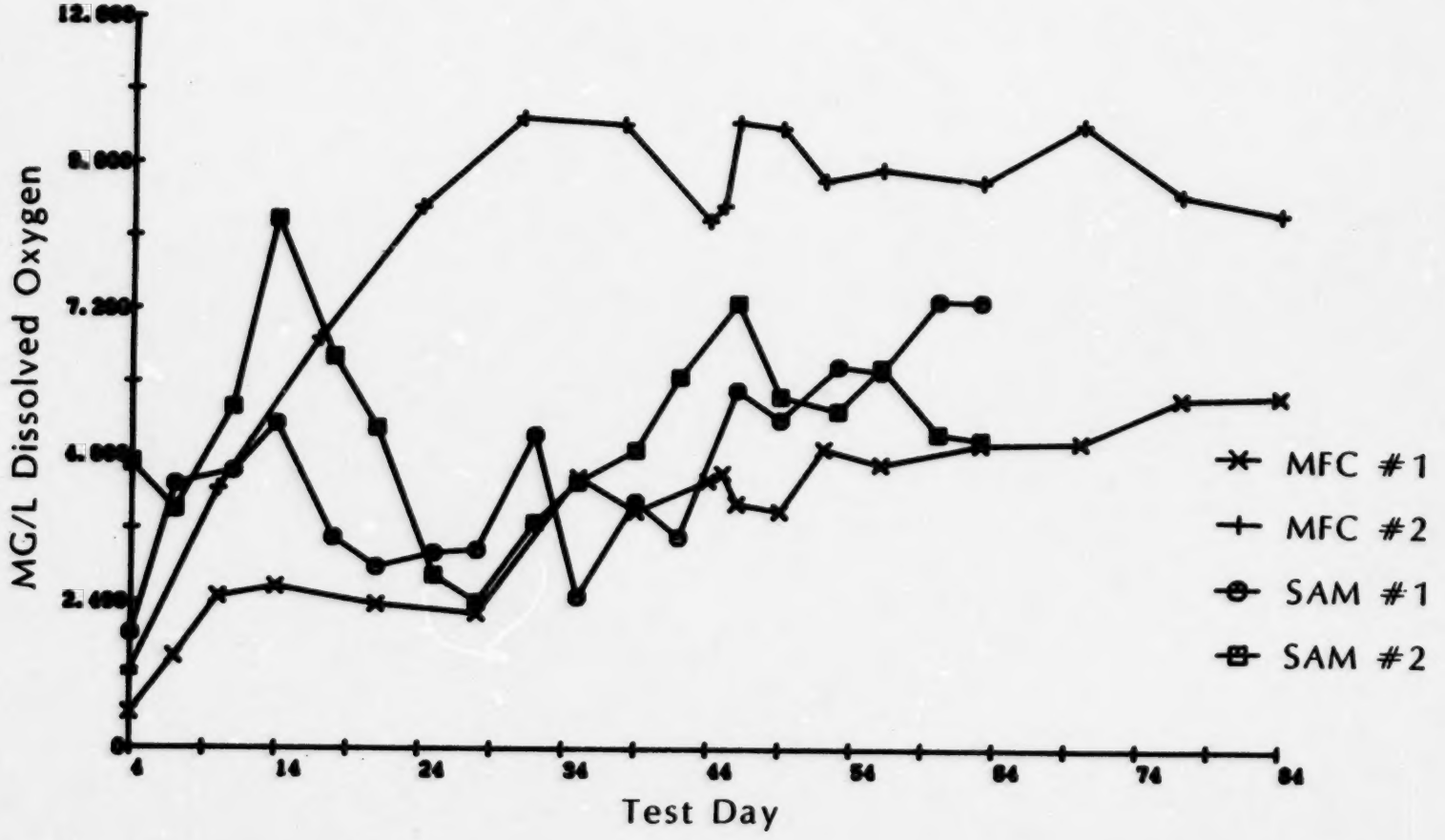

MG/L Dissolved Oxygen
12.000
8.000
7.200
4.000
2.400
0
4
14
24
34
44
54
64
74
84
Test Day
MFC #1
MFC #2
SAM #1
SAM #2

Table 1- Comparison of Mixed Flask Culture (MFC) and Standardized Aquatic Microcosm (SAM) protocols.

	MFC	SAM
Microcosm volume:	1 liter	3 liters
Inoculation:	Set volume of mixed lab culture	Set density of 15 species
Pre-treatment period:	6 weeks	1 week
Post-treatment period:	6 weeks	8 weeks
Reinoculation:	50 ml of mixed lab culture weekly.	Missing zooplankton species reintroduced, low numbers of other species added.
Observations made:	Dissolved oxygen, pH, Eh.	Species densities, oxygen, pH, nutrients, fluorescence.
Test design:	7 treatment levels, 5 replicates	4 treatment levels, 6 replicates
Data obtained: (after treatment)	12 parameters on 10 dates	40-43 parameters on 16 dates (nutrients sampled less often)

Table 2- Regression analysis of the effects of copper on oxygen gain in MFC and SAM microcosms. Data is from the the first monitoring day following treatment. Oxygen gain was regressed against the log of copper concentration + 1.

	MFC #1	MFC #2	SAM #1	SAM #2
n of cases	30	35	24	24
slope (b)	-1.090	- .737	-1.421	-1.401
intercept (a)	5.21	6.48	4.58	5.56
r2	.691	.404	.975	.852
F	62.5*	22.3*	881.5*	126.8*

* significant at $p < .001$

Table 3- Comparison of Coefficients of Variation and Minimum Detectable Differences.

Coefficients of variation (CV) and Minimum Detectable Differences (MDD) calculated from control microcosm data during post-treatment observations. The means from all observations are shown for each experiment done under the Mixed Flask Culture (MFC) protocol, and the Standardized Aquatic Microcosm (SAM) protocol.

	Dissolved Oxygen AM1	Dissolved Oxygen PM	Dissolved Oxygen AM2	All Oxygen Obs.	pH AM
	CV	CV	CV	CV / MDD	CV / MDD
MFC #1	2.86	2.74	3.02	2.87/.0614	2.37/.0507
MFC #2	8.28	5.85	8.59	7.57/.1609	2.30/.0489
Mean:	5.57	4.29	5.80	5.22/.1110	2.34/.0497
SAM #1	10.86	9.78	10.11	10.25/.2255	2.90/.0638
SAM #2	17.43	16.94	18.58	17.65/.3883	2.90/.0638
Mean:	14.14	13.36	14.34	13.95/.3069	2.90/.0638

Table 4- Comparison of time requirements for running tests with MFC and SAM protocols.

Activity	Time Required (hours)	
	MFC	SAM
Preparation and Setup	5	21
Pre-dose Monitoring	4.5	40
Cull,add Test Chemicals	2.5	2
Post-dose Monitoring	43.50	271.8
Nutrient Analysis	-	62.4
Weekly Reinoculation	3.6	8.0
Cross-inoculation	1.3	-
Check light intensity	-	1.8
Rotate Microcosms	3.6	-
Culture Maintainance	3	18
Data Analysis	variable	variable
Total	67.00	425.0

Bibliography

[1] Giesy,J.P. Jr.(ed). Microcosms in Ecological Research. DOE Symposium Series 52. 1110pp. 1980.

[2] National Academy of Sciences, Committee to Review Methods for Ecotoxicology.Testing for Effects of Chemicals on Ecosystems.National Academy Press, Washington, DC. 103 pp. 1981.

[3] Hammons, A.S. (ed.). "Methods for Ecological Toxicology: a critical review of laboratory multispecies tests." ORNL-5708, EPA-560/11-80-026. Ann Arbor Science, Ann Arbor, MI 1981.

[4] Cairns, J.,Jr. Are single species toxicity tests alone adequate for estimating environmental hazard? Hydrobiologia 100:47-57. 1983.

[5] Levin, S.A. et al.(ed). New Perspectives in Ecotoxicology. Environmental Management 8(5):375-442. 1984.

[6] Smies, M. On the relevance of microecosystems for risk assessment: Some Considerations for Environmental Toxicology. Ecotoxicology and Environmental Safety 7:335-365. 1983.

[7] Taub. F.B. and P.L.Read. "Standardized aquatic microcosm protocol." Final Report, Vol. II of FDA Contract 223-80-2352. 1982.

[8] Leffler, J.W. "Tentative Protocol of an Aquatic Microcosm Screening Test for Evaluating Ecosystem-level Effects of Chemicals." Developed under Battelle Columbus Laboratories Subcontract #T6411 (7197), Ferrum College, Ferrum, Va. 1981.

[9] McConnell,W.J.. "Productivity relations in carboy microcosms." Limnol.and Oceanogr. 7:335-343. 1962.

[10] Strickland,J.D.H., and T.R. Parsons. A practical handbook of seawater analysis. 2nd Ed. Bull. Fish Res. Bd. Can. 167, 310 pp. 1972.

[11] Wetzel,Robert. Limnology W.B.Saunders,Chicago. p299. 1983.

[12] Steel,R and Torrie. Principles and Procedures of Statistics. McGraw Hill New York,NY. p111. 1960.

[13] Taub, Frieda B., A.F. Kindig, P.T. Munro, and L.L. Conquest. "Model Ecosystems: Effects of chelate concentration on copper toxicity." Experiment report ME 57. FDA Contract 223-80-2352. 120p. 1982.

[14] Sokal, Robert R. and James Rohlf. Biometry. W.H. Freeman and Co., San Francisco. p448. 1969.

[15] Conquest,Loveday L.. "Assessing the statistical effectiveness of ecological experiments: Utility of the coefficient of variation." Intern. J. Environ. Stud. 20: 209-221. 1983.

[16] Harrass M.C. and F.B.Taub, "Comparison of laboratory microcosms and field responses to pesticides." ASTM Special Technical Publication. American Society for Testing and Materials, Philadelphia. In Press.

[17] Kindig, A.C. "Interactions between algal nutrient availability and an algicide in aquatic microcosm communities." Ph.D. Dissertation, University of Washington, Seattle. 259p. 1982.

[17] Hardy, F.J. Ph.D. Dissertation, University of Washington, Seattle. 1984.

[18] United States Environmental Protection Agency. "Ambient aquatic life water quality criteria for copper." Draft Report. 1985

[19] McKnight, D.M. and F.M.M. Morel. "Release of weak and strong copper-complexing agents by algae." Limnol. Oceanogr. 24:823-837. 1979.

[20] Swallow, K.C., J.C. Westall, D.M. McKnight, N.M. Morel, and F.M. Morel. "Potentiometric determination of copper complexation by phytoplankton exudates." Limnol. Oceanogr. 23: 538-542. 1978.

[21] Adema, D.V.M. and A.M. Degroot-Van Zijl. "The influence of copper on the water flea Daphnia magna." TNO Nieuws. 27: 474- 481. 1972.

[22] Winner, R.W. and M.P. Farrell. "Acute and chronic toxicity of copper to four species of Daphnia." Jour. Fish Res. Board Can. 33: 1685-1691. 1976.

[23] Steeman-Nielsen, E. and S. Wium-Andersen. "Copper ions as poison in sea and freshwater." Mar. Biol. 6: 93-102. 1970.

[24] Cairns, John Jr., et al "Effects of temperature on aquatic organism sensitivity to selected chemicals". Bulletin 106. Virginia Water Resources Resources Center, Blacksburg, Virginia. 1978.

[25] Giddings, J.M. "Microcosms for assessment of chemical effects on the properties of aquatic ecosystems". Hazard Assessment of Chemicals: current developments Vol. 2: 45-94. 1983.

Footnote

1 Kindig,Andrew. personal communication

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